Chronic Myelocytic Leukemia (CML) Patient-Derived Dendritic Cells Transfected with Autologous Total RNA Induces CML-Specific Cytotoxicity

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Abstract The oncogenic bcr/abl1 fusion gene is a chronic myelogenous leukemia (CML)-specific antigen which is absent in normal tissues. This makes bcr/abl1 a perfect target for developing CML vaccines that elicit specific immune responses against minimal residual disease while sparing normal tissue. The aim of this study was to use different methods to induce dendritic cells (DCs) derived from patients with CML (CML-DCs) and analyze them for CML-specific tumor cytotoxicity for immune therapy. Bone marrow-derived mononuclear cells from ten CML patients were studied to induce CML-DC differentiation in the presence of recombinant human interleukin-4, rh-granulocyte-macrophage-colony stimulating factor, and tumor necrosis factor-alpha with either a total RNA-lipofectamine complex, total RNA or CML tumor lysate (freeze-thawed). CML-DC maturation, confirmed by expression of CD1 α , CD40, CD80, CD83, CD86 and by real-time polymerase chain reaction, validated the CML-origin of these DC cells. CML-DCs stimulated cytotoxic T-cell (CTL) apoptosis, high levels of IL-12 secretion, and had significant inhibitory effect on K562 tumorigenicity in nude mice. CML-DCs pulsed with total RNA by lipofectamine transfection produced the strongest effect in tumor-specific CTL functions. These results indicate that CML-DCs transfected with total RNA by lipofectamine induce the strongest CTL cytotoxicity and have the greatest potential for CML immune therapy. This study holds

promise for a DC-based strategy for inducing anti-leukemia responses and establishes a foundation for developing RNA vaccination against CML.

Keywords Dendritic cell · Chronic myelocytic leukemia · Total RNA · Lipofectamine transfection

Introduction

The translocation t(9;22) and expression of bcr/abl1 fusion protein is unique in chronic myeloid leukemia (CML). Although tyrosine kinase inhibitors (TKIs), such as imatinib mesylate and dasatinib, are standard treatments for CML, highly sensitive molecular techniques can still detect minimal residual disease (MRD) in treated patients [1–3]. A small percentage of these patients will develop resistance to this therapy and result in relapse. Allogeneic hematopoietic stem cell transplantation (aHSCT) is the only remaining option capable of bringing relapsed CML patients back to remission [4]. This has been demonstrated by the curative potential of allogeneic stem cell transplantation (SCT) and the re-induction of complete remission upon donor lymphocyte infusion in patients with relapsed CML [4, 7].

The oncogenic driver of CML, bcr/abl1, is not only the target of TKIs, but also a CML-specific antigen absent in normal tissues, making it a perfect target for developing CML vaccines that elicit specific immune responses against MRD, while sparing normal tissue [5, 6]. CML patients have significantly lower numbers of circulating myeloid and plasmacytoid dendritic cells (pDCs) compared with healthy volunteers [8, 9]. Bcr/abl1-presenting DCs have been detected in the peripheral blood of CML patients, suggesting that CML-derived DCs may contribute

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to anti-leukemic immunity [10, 11]. Bcr/abl1-presenting DCs can be generated from peripheral blood mononuclear cells (PBMCs) or CD34c progenitor cells of CML patients, and have been shown to have the capacity to capture, process and present bcr/abl1 antigen [5, 12]. Active and specific immunotherapy targeting of the bcr/abl1-derived p210 fusion protein improves patient outcome and it has recently been reported that CML patients have similar immunological responses to the e14a2 bcr/abl1 peptide as healthy subjects under the same vaccination schedule [13, 14]. Therefore, strategies employing active and specific DC-based vaccination have the potential to eradicate MRD when applied in combination with TKIs, or as an alternative treatment for patients resistant to TKI therapy.

Based on these encouraging reports, there is an urgent need to develop effective methods for inducing the number of DCs with improved function in CML patients. Our study demonstrated that DC vaccination by CML tumor antigen and DC transfection with total CML RNA produced T-cells with anti-tumor effect in vitro and in vivo. The lipofectamine-mediated transfection of total CML RNA induced the strongest cytotoxic T-cell (CTL) cytotoxicity. Our results provide an experimental basis for the development of molecular immune therapy for CML.

Methods

Patient Samples and Animals

Samples from ten cases of newly diagnosed, chronic phase, CML patients (before treatment) were collected at our hospital (seven males and three females), with an age range from 26 to 70 years and median age of 46 years. All samples tested positive for the Ph1 chromosome or bcr–abl1 fusion gene. The clinical study was approved by the Medical Ethics Committee of the Second Affiliate Hospital to Nanchang University and all patients provided written informed consent to participate in the study.

Male BALB/c mice, 6–8 weeks of age, were purchased from the Guangdong Provincial Medical Experimental Animal Center (animal certificate No. SCXK2008-0002), and were bred at the Experimental Animal Center of Medicine College to Nanchang University under controlled conditions. All animals received standard laboratory chow and water according to institutional guidelines. Upon delivery, the mice were allowed to acclimatize for 1–2 weeks before the start of the experiment. Experiments were approved by the local Animal Ethics Committee and followed international ethical standards of conduct.

Preparation of CML Tumor Freeze–Thawed Antigen, Total RNA, and Total RNA with Lipofectamine for Transfection

Bone marrow mononuclear cells (BMMNCs, 10^7 /ml) from CML patients were collected, separated and subjected to three freeze/thaw cycles (room temperature and $-70\text{ }^{\circ}\text{C}$), prior to centrifugation at $3500\times g$ for 10 min. The supernatant was used as the freeze–thawed antigen from the CML tumor. Total RNA from BMMNCs of CML patients was extracted by Trizol using the standard protocol described by the manufacturer and quantified using a nucleic acid protein analyzer (ScanDrop 200, Analytik Jena AG, German). Total RNA (10 μg) and Lipofectamine 2000 (10 μl , Invitrogen, Waltham, MA, USA) were each diluted with 500 μl of serum-free RPMI1640 culture medium and left at room temperature for 5 min. Samples were then mixed and incubated for 30 min at $37\text{ }^{\circ}\text{C}$ with 5 % CO_2 in a CO_2 incubator (SHELL/JB, Cornelius, OR, USA) to generate the total RNA-lipofectamine complex for transfection.

CML-DC Culture and Loading of CML Tumor-Derived Antigen into Immature DCs

For the CML-DC culture system, 1×10^6 BMMNCs were cultured in IMDM (Iscoe's Modified Dulbecco's Medium) containing 10 % calf serum, 100 ng/ml granulocyte-macrophage-colony stimulating factor (GM-CSF), and 100 ng/ml interleukin-4 (IL-4, Cytolab, Ceder, Utah, USA), at $37\text{ }^{\circ}\text{C}$ in 5 % CO_2 with saturated atmospheric humidity. Half of the medium was replaced by fresh media on the 3rd day. On the 5th day, different antigens were added to the DC culture: (1) freeze–thawed antigen from the CML tumor; (2) total RNA; and (3) total RNA-lipofectamine complex for transfection. The total RNA-lipofectamine complex was added to the DC culture and incubated for 4 h at $37\text{ }^{\circ}\text{C}$ with 5 % CO_2 . Afterwards, the supernatant was replaced with fresh culture media in accordance with the DC culture method. Tumor necrosis factor-alpha (TNF- α , 10 ng/ml, Cytolab) was added on the 7th day and mature DCs were harvested on the 14th day. Cell morphology was examined daily under an inverted phase contrast microscope.

Five groups of cells were prepared for subsequent assays as follows: (1) DCs transfected with total CML RNA-lipofectamine; (2) DCs incubated with total CML RNA; (3) DCs incubated with freeze–thawed CML antigen; (4) untreated control CML-DCs; and (5) lymphocytes stimulated by IL-2.

Flow Cytometry

Antibodies against CD1 α , CD40, CD80, CD83, and CD86 (eBioscience, San Diego, CA, USA) were used to monitor DC cultures by flow cytometry (FACSCalibur four-color flow cytometer, BD Biosciences, Seattle, WA, USA). As previously described [15], 5 μ l of the appropriate monoclonal antibody was incubated with 100 μ l of CML-DCs (1×10^6 /ml) for 15 min in the dark followed by resuspension in phosphate buffer saline (PBS, pH 7.4) for analysis.

Real-Time Polymerase Chain Reaction (RT-PCR) Detection of bcr-abl1 Fusion Genes in CML-DCs

The primers used in the present study were as follows: bcr/abl1 FORWARD: 5-ATTCTCGAGATGGGGCTCTATGGTTTCTG-3, and REVERSE: 5-GCCGAATTCCTAGATGTAGTTGCTTGGGAC-3.

The protocol used for RT-PCR was carried out as follows: 1st stage: 95 °C for 4 min; 2nd stage: 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; 3rd stage: one cycle at 72 °C for 10 min. PCR products (5 μ l) were loaded into the agarose gel alongside β -actin and a molecular weight marker as controls. Electrophoresis was conducted at 100 V for 30 min. The results were observed and captured using a gel imaging system.

Cytotoxicity Assay

Adherent CML mononuclear cells (5×10^6) were cultured in the presence of IL-2 (100 ng/ml) and inoculated with mature CML-DCs (generated by different treatments) at a ratio of 3:1 on the 7th day. The resulting CTL cells (effector cells) were formed on the 14th day, as confirmed by CD3 expression using flow cytometry.

CML mononuclear cells from patients were thawed and cultured in autologous serum for 72 h. These cells were used as target cells for monitoring cytotoxicity by mixing with effector CTL cells at a 20:1 ratio, and were plated into 96-well culture plates. Cultures comprising target cells only and effector cells only were used as controls. The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to determine the level of cell survival after 24 h of incubation. MTT (20 μ l) was added to each well and incubated for 4 h. After removing the supernatant, dimethyl sulfoxide (DMSO) was added to dissolve the MTT. A microplate reader was used to read the absorption value at a wavelength of 570 nm. The experiment was repeated three times for statistical analysis.

$$\text{CTL activity (\%)} = \frac{[(\text{absorbance of the target cell only} - \text{absorbance of the experimental well} + \text{absorbance of the effector cell only}) / \text{absorbance of the target cell only}] \times 100 \%}{}$$

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-12 concentrations were determined by sandwich ELISA kits (Bender Medsystems, Austria) using a BIO-RAD model 450 ELISA plate reader (BIO-RAD, Hercules, CA, USA) to measure the optical density at 450 nm. A standard curve was generated by plotting the mean absorbance of IL-12 at defined concentrations. IL-12 concentration for each sample was expressed in nanograms per milliliter as determined by the standard curve. This kit was able to detect IL-12 P40/70 at concentrations as low as 3.96 ng/ml.

Animal Study

A total of 25 male BALB/c mice were divided into five groups and 5 Gy irradiation was given to each mouse, followed by cell inoculation within 2 h after the radiation. K562 cells (5×10^6) were used as target cells for determining tumor-specific CTL cytotoxicity. The ratio of the CML-DCs to the target cells was 5:1 reconstituted into a 0.2 ml final volume. Cells were injected subcutaneously at the right root of the hind legs near the back of the nude mice. The mice were sacrificed 8 weeks after injection and tumors were extracted. The tumors were weighed and the rate of tumor growth/inhibition was calculated by comparing with the blank control group. A portion of the tumor tissues was fixed in formalin, dehydrated by ethanol, and sectioned onto slides. The sections were stained with hematoxylin and eosin (HE) and mounted in balsam. Cell morphology was visualized under an inverted light microscope.

Statistical Analysis

Data are presented as the mean $\pm$ standard deviation (SD) and differences between groups were analyzed using the *t* test. All statistical analyses were performed with SPSS 16.0 (SPSS Inc., USA). The difference was considered to be statistically significant at $p < 0.05$.

Results

Morphological Changes of CML-DCs

The freshly separated CML cells were uniform, spherical cells with smooth surfaces. After 3 days in suspended

culture, they proliferated into clusters and a small number of cells developed minor protrusions. On the 8th day, some cells appeared to have irregular morphology generating burr-shaped protuberance. Most cells showed a typical DC morphology with a large number of burr-shaped protrusions in a semi-suspension state on the 14th day of culture.

Immunophenotype of CML-DCs

To confirm maturation of DCs derived from CML patient BMMNCs in culture, the expression of mature DC markers CD1 α , CD40, CD80, CD86, and CD83 were examined by flow cytometry. Expression of all DC maturation markers were significantly higher in cultured BMMNCs (CML-DCs) than those before culture (PBMNC, $p < 0.01$). With the exception of CD86, positive expression levels of DC maturation markers were significantly elevated in DCs inoculated with CML-derived antigen (freeze/thawed, total RNA, and RNA-lipofectamine) compared with control CML-DCs (Fig. 1). Intriguingly, the total RNA-lipofectamine group resulted in the highest induction of CD40 and CD83 expression in CML-derived DCs.

Bcr/abl1 Fusion Genes and Total RNA in the Mature CML-DCs

Electrophoresis with total RNA identified two clear bands of 18 s and 28 s with a ratio of A260/A280 ranging from 1.8 to 2.0, indicating that the extracted RNA was not degraded and had good purity (Fig. 2a). Two forms of the bcr/abl1 fusion gene exist: e14a2 (165 bp) and e13a2 (90 bp), and their presence in the CML-derived DCs can confirm their CML origin. RT-PCR detected both forms of bcr/abl1 fusion gene, as well as the positive β -actin control (Fig. 2b, c).

Comparison of the CTL Cytotoxicity Stimulated by CML-DCs from Different Groups

Maturation of the cultured CTL cells was determined by CD3 expression level, which was 73.21 ± 2.52 % for all samples. The cytotoxicity assay showed that at the target/effector cell ratio of 20:1, the cytotoxicity rates of CTLs stimulated by CML-DC inoculated with total RNA, total RNA-lipofectamine, or freeze-thawed antigen had significantly higher cytotoxicity levels than the control groups (PBMNCs and CML-DCs) as shown in Table 1. All the pulsed groups had statistically significantly higher cytotoxicity levels compared with the blank control group (PBMNC, $p < 0.001$, Table 1).

IL-12 Levels

The CTLs stimulated by DCs pulsed with total RNA-lipofectamine, total RNA, and freeze-thawed antigen, had a significantly higher IL-12 level compared with the CML-DC group ($p < 0.05$, Table 1). Cultured CML cells did not secrete any IL-12 (data not shown).

Tumor Volumes and Rates of Growth/Inhibition Among Different Experimental Groups

K562 cells (5×10^6), used as target cells, were mixed with CML-DCs and injected subcutaneously at the right root of the hind legs near the back of nude mice. The tumor growth and progression results demonstrated tumor-specific CTL cytotoxicity, which was mediated by DCs pulsed with CML-derived antigens. Significant reduction in tumor volume and delayed tumor latency were observed in the groups co-injected with CTLs activated by CML-DCs pulsed with (1) freeze-thawed tumor lysate; (2) total RNA; and (3) total RNA-lipofectamine; in comparison with unstimulated CML-DC control. Total RNA-lipofectamine inoculation produced CML-DCs with an elevated ability to induce tumor-specific CTLs, resulting in the smallest tumor volume (80 mm^3) and the highest growth inhibition of 50 % (Table 1). Total RNA and freeze-thawed tumor lysates were at equal efficacy with 35 % growth inhibition and tumor volumes of 100 mm^3 . Unstimulated CML-DC control cells had a weak effect with a tumor volume at 150 mm^3 and 24 % growth inhibition. In the absence of DC inoculation, maximum tumor size was reached at 200 mm^3 (Table 1). While there was a trend for delayed latency of tumor formation in the DC-treated groups compared with the PBMNC control, there were no significant changes in latency produced by the different types of antigen inoculation.

Pathological Observation of Tumor Tissues

Tumors injected with K562 cells were extracted, fixed and sectioned for pathological observation. Histological analysis confirmed that the tumors were leukemia sarcoma. All tumor tissues showed loss of structural integrity to some extent and had similar pathological characteristics. Histological sections demonstrated a mixed cellular infiltrate featuring immature myeloid cells, lymphocytes, and macrophages with partial necrosis area. Larger infiltrating cells with atypical nuclei were also observed in the mixture. Mild-to-moderate infiltrations by K562 cells were observed in large areas of skin, muscles and other tissues around the tumor tissues (Fig. 3).

Fig. 1 Flow cytometric analysis of cell groups for cell surface markers. **a** Flow cytometric *dot plots* of BMMNC-derived DCs before (1–3) and after (4–6) being pulsed with total RNA-lipofectamine. **b** Immunophenotype of BMMNC-derived DCs pulsed with different

antigens from CML cells compared with untreated PBMNCs. Expression levels (%) of CD1 α , CD83, CD40, CD80, and CD86 are shown. *PBMNC* peripheral blood mononuclear cells, *CML* chronic myeloid leukemia, *DC* dendritic cell, *NA* not available

Fig. 2 Bcr/abl1 fusion genes in mature CML-DCs compared with total RNA of CML-BMMNCs. **a** CML-BMMNC total RNA showing two clear bands of 18 and 28 s. **b** b3a2 showing a 165 bp expression product. **c** 2a2 showing a 90-bp expression product

Table 1 Cytotoxic T-cell (CTL) % from CML-DCs, IL-12 level, tumor formation time and mass, and tumor growth inhibition rate for different groups

Group	CTL (%)	IL-12 (pg/ml)	Tumor formation time (days)	Tumor mass volume (mm ³)	Inhibition rate
PBMNC	10.62 ± 7.17	0	24.12 ± 4.56	208.39 ± 14.59	NA
CML-DC	31.52 ± 1.82**	63.5 ± 3.6	30.67 ± 4.95	156.87 ± 13.34*	24.24 ± 4.95
RNA-lipofectamine	74.67 ± 5.76**	132.8 ± 11.5*	30.94 ± 5.42	82.21 ± 11.62**	50.67 ± 6.64**
Total RNA	36.45 ± 3.02**	105.7 ± 15.6*	31.43 ± 3.78	102.08 ± 12.75**	35.87 ± 4.97*
Tumor lysate	51.07 ± 5.34**	89.6 ± 9.5*	32.21 ± 3.2	109.68 ± 16.54**	33.54 ± 3.78*

Tumor growth inhibition rates were calculated as the ratio of the experimental group compared with the PBMNC control group

PBMNC peripheral blood mononuclear cell, CML chronic myeloid leukemia, DC dendritic cell, NA data not available

* $p < 0.05$; ** $p < 0.001$

Discussion

CML patients have severe immune dysfunction and significantly reduced numbers of DCs [12, 16]. In a paper by Eisendle et al. [14], it was reported that the immature CML-DCs had decreased pinocytotic ability (hence impaired antigen processing and presentation), and even matured CML-DCs had defects in antigen-presentation function with decreased expression of CD80, CD83, CD86, MHC Class I, and MHC Class II molecules. This study demonstrated an alternative method to load leukemia antigens into CML-DCs and enable them to induce strong CML-specific CTL cytotoxicity.

We investigated different methods to inoculate CML-DCs for vaccine preparation, including: (1) total RNA-lipofectamine complex for transfection; (2) total RNA; (3) tumor lysate (antigen) by freeze-thawed CML cells; (4) unpulsed CML-DCs and (5) control PBMNCs. The CML-DC functions induced by these five groups were evaluated by CTL cytotoxicity, IL-12 level, and inhibition of tumorigenicity in nude mice. The results showed that CML-DCs transfected with total RNA by lipofectamine had the strongest DC function including greatest CTL cytotoxicity, highest IL-12 level, and largest reduction in tumor volume.

The leukemia-DC vaccine is an immune therapeutic strategy in which DCs are induced, amplified, and loaded with leukemia antigens, to generate a strong anti-tumor immunoreaction. The anti-tumor effect of this type of vaccine makes it extremely valuable for clinical application [17, 18]. However, the methods used for loading antigens affect CTL functions and may lead to undesirable side effects. Inoculation with CML tumor lysate produces an enhancement in CML-DC antigen-presenting function [19–21], but it may also induce autoimmune diseases when used in humans [12]. DNA-loaded DCs using constructed plasmids could induce specific CTL reactions [21], but these plasmids present a potential risk because they may persist in DCs for months by integrating into the genome or remaining active as unintegrated DNA in non-dividing cells [11, 22–24]. RNA-loaded DCs can generate specific CTL effects and induce polyclonal CD8+ and CD4+ CTLs by targeting different epitopes [25–27]. The RNA vaccine is relatively safe owing to a short half-life and low possibility of genome integration. Furthermore, using total RNA for vaccination does not require the identification of specific tumor antigens and construction of plasmids, making RNA vaccines easily adaptable for clinical use [28]. Consequently, for developing a CML-specific immune therapy, we focused on the RNA-based method to

Fig. 3 Pathological observation of the tumor tissues (total RNA-lipofectamine group). **a** Tumor node of the nude mice. **b** Low power view of an involved tumor tissue with HE staining showing mild-to-moderate infiltration by K562 cells was in a large area of skin, muscles, and other tissues around the tumor tissues (*arrow*). **c**. Higher magnification reveals typical features of the necrosis area (*white arrow*) and K562 cell infiltration (*black arrow*). **a** Pathological appearance, **b** magnified $\times 10$, **c** magnified $\times 40$

inoculate CML-DCs, comparing this approach with freeze-thawed CML lysates.

Uptake of RNA from the surrounding environment by DCs for antigen processing depends on the ability of immature DCs to perform phagocytosis. CML-DCs have been shown to internalize small amounts of total RNA through phagocytosis. Other than incubating DCs directly with RNA, additional methods are available for inducing the internalization of RNA by DCs, including RNA-lipofectamine transfection, adenovirus infection, and electroporation [29]. From our study, CML-DCs incubated with total RNA had only weak CTL induction compared with those inoculated with total RNA-liposomal complexes. These results indicated that incubation of DCs with naked RNA from CMLs could not produce sufficient responses for immune therapy and a lipofectamine carrier is required to boost RNA uptake efficiency. The use of cationic lipofectamines as carriers for RNA vaccine has been widely accepted and the technique is becoming increasingly popular [30]. However, the optimal RNA dosage needed for efficient transfection varies among reports, ranging from 1 to 30 μg per million DCs [31, 32]. In our experiments, we used 10 μg total RNA per million DCs for transfection and have successfully vaccinated CML-DCs for antigen presentation. Further refinement will be needed to achieve an optimal balance between RNA and lipofectamine to minimize liposomal toxicity and maximized transfection efficiency.

In this study, we demonstrated that CML-DCs derived from patients have the ability to process and present CML-specific antigens by inoculation with total RNA-lipofectamine complex, naked total RNA, and freeze-thawed tumor lysate. These activated CML-DCs induced CTLs for elevated and CML-specific cytotoxic responses compared with unstimulated CML-DCs and control PBMNCs. Most importantly, total RNA transfection by lipofectamine was proven to be the most effective method for delivering RNA vaccine and resulted in the strongest CTL cytotoxicity. Therefore, our data highlight the potency of a DC-based strategy for inducing anti-leukemia responses and establish a foundation for developing RNA vaccination against CML. Further investigations using this approach have the potential to revolutionize CML therapy by molecular immune vaccination.

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